ACTH stimulates insulin secretion from MIN6 cells and primary mouse and human islets of Langerhans

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Abstract

It has previously been suggested that ACTH and ACTH-related peptides may act as paracrine modulators of insulin secretion in the islets of Langerhans. We have, therefore, examined the expression and function of the ACTH receptor (the melanocortin 2 receptor, MC2-R) in human and mouse primary islet tissue and in the MIN6 mouse insulinoma cell line. Mouse MC2-R mRNA was detected in both MIN6 cells and mouse islet tissue by PCR amplification of cDNA. In perifusion experiments with MIN6 pseudo-islets, a small, transient increase in insulin secretion was obtained when $ACTH_{1-24}$ (1 nM) was added to medium containing 2 mM glucose (control) but not when the medium glucose content was increased to 8 mM. Further investigations were performed using static incubations of MIN6 cell monolayers; $ACTH_{1-24}$ (1 pM–10 nM) provoked a concentration-dependent increase in insulin secretion from MIN6 monolayer cells that achieved statistical significance at concentrations of 1 and 10 nM ($150 \pm 13{\cdot}6\%$ basal secretion; $187 \pm 14{\cdot}9\%$ basal secretion, $P < 0{\cdot}01$). Similar responses were obtained with $ACTH_{1-39}$. The phosphodiesterase inhibitor IBMX (100 μM) potentiated the responses to sub-maximal doses of $ACTH_{1-24}$. Two inhibitors of the protein kinase A (PKA) signaling pathway, Rp-cAMPS (500 μM) and H-89 (10 μM), abolished the insulin secretory response to $ACTH_{1-24}$ (0·5–10 nM). Treatment with 1 nM $ACTH_{1-24}$ caused a small, statistically significant increase in intracellular cAMP levels. Secretory responses of MIN6 cells to $ACTH_{1-24}$ were also influenced by changes in extracellular Ca^{2+} levels. Incubation in Ca^{2+}-free buffer supplemented with 0·1 mM EGTA blocked the MIN6 cells' secretory response to 1 and 10 nM $ACTH_{1-24}$. Similar results were obtained when a Ca^{2+} channel blocker (nitrendipine, 10 μM) was added to the Ca^{2+}-containing buffer.

$ACTH_{1-24}$ also evoked an insulin secretory response from primary tissues. The addition of $ACTH_{1-24}$ (0·5 nM) to perifusions of mouse islets induced a transient increase in insulin secretion at 8 mM glucose. Perifused human primary islets also showed a secretory response to $ACTH_{1-24}$ at basal glucose concentration (2 mM) with a rapid initial spike in insulin secretion followed by a decline to basal levels. Overall the results demonstrate that the MC2-R is expressed in β-cells and suggest that activation of the receptor by ACTH initiates insulin secretion through the activation of PKA in association with Ca^{2+} influx into β-cells.

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Introduction

It has been known for many years that adrenocorticotropin (ACTH) not only plays an essential role in the control of the adrenal cortex but that it also influences a number of tissues outside the gland including the central nervous system, adipose tissue and skin (see Hadley & Haskell-Luevano 1999, Solomon 1999). Following reports that treatment with ACTH caused hypoglycemia it was suggested that the endocrine pancreas might also be a target (Lebovitz *et al.* 1965, 1966). This view gained further support from the demonstration that ACTH directly stimulated insulin secretion by pancreatic preparations from a range of animals including rats, mice, rabbits and toads (Lebovitz & Pooler 1967, Malaisse *et al.* 1967, Sussman & Vaughan 1967, Curry & Bennet 1973, Flores *et al.* 1998). More recent studies have confirmed that exogenous ACTH also enhances the secretion of insulin by isolated rat islets of Langerhans (Borelli *et al.* 1994, 1996, Gagliardino *et al.* 1995, 1997).

The physiological effects of ACTH in the adrenal cortex are mediated through the melanocortin 2 receptor (MC2-R) which is expressed at high levels in the gland and coupled to the cAMP/protein kinase A (PKA) signal transduction system (Mountjoy *et al.* 1992, Vinson *et al.* 1992, Xia & Wikberg 1996). In agreement with this, early

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studies of the effects of ACTH on β-cells suggested the involvement of cAMP, although these generally used non-physiological concentrations of ACTH (Malaisse *et al.* 1967, Sussman & Vaughan 1967, Kuo *et al.* 1973). More recently intracellular Ca^{2+} has been identified as a key regulator of insulin secretion (see Ashcroft & Ashcroft 1992) and changes in intracellular Ca^{2+} have also been implicated in the stimulatory effects of ACTH on insulin secretion from mouse β-cells (Gronda *et al.* 1992, Gagliardino *et al.* 1995, 1997).

In the present study we have examined the expression and function of the MC2-R in primary islets and an insulin-secreting β-cell line to determine whether physiologically relevant concentrations of ACTH can influence β-cell function, and to identify the intracellular transduction mechanism involved. We demonstrate that the MC2-R is expressed in β-cells and that activation of the receptor by ACTH initiates insulin secretion through the activation of PKA in association with Ca^{2+} influx into the β-cells.

Materials and Methods

Materials

MIN6 cells were kindly provided by Professor J I Miyazaki (University of Osaka, Japan). PCR primer preparation and DNA sequencing was performed by the Molecular Biology Unit, King's College London, UK. Connaught Medical Research Laboratory (CMRL) medium, fetal calf serum (FCS), glutamine, penicillin/streptomycin and Superscript II reverse transcriptase were obtained from Gibco (Paisley, UK). Taq DNA polymerase was purchased from Promega, RNAzol B from Biogenesis (Poole, UK), Rp-cAMPS (adenosine-3′,5′-cyclic monophosphorothioate) from BIOLOG (Bremen, Germany) and H-89 from Calbiochem (Nottingham, UK). $ACTH_{1–24}$ (tetracosactrin acetate; Synacthen) was supplied by Alliance Pharmaceticals, Chippenham, UK). All other biochemicals were obtained from Sigma.

Maintenance of MIN6 cells and pseudoislets

MIN6 cells were maintained at 37 °C (95% O_2/5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FCS, 2 mM glutamine and 100 U/ml penicillin/0·1 mg/ml streptomycin. Medium was changed every 3–4 days and monolayer cells were detached from the tissue culture plastic using a 0·02% EDTA solution when 80–90% confluent. MIN6 pseudoislets were cultured under the same conditions as monolayers but in tissue culture flasks pre-coated with gelatin (1% w/v). All experiments using pseudoislets were carried out 6–8 days after subculturing of the MIN6 cells.

Isolation of human and mouse islets

Mouse islets of Langerhans were prepared by collagenase digestion of the pancreas *in vitro*, and purified by hand-picking under a dissecting microscope, as described previously (Roderigo-Milne *et al.* 2002). Human islets were obtained through the Dixon's Human Islet Project at King's College Hospital. Pancreata were retrieved, with permission, from non-diabetic, heart-beating organ donors and islets were isolated under aseptic conditions according to the method of London *et al.* (1990, 1998). Islets were used after maintenance overnight in CMRL medium supplemented with 15% FCS and 100U/ml penicillin/0·1 mg/ml streptomycin.

Insulin secretion

For static incubation experiments, MIN6 monolayer cells were seeded into 96-well plates at a density of 30 000 cells/well and cultured for 2 days in DMEM at 37 °C. The cells were pre-incubated in glucose-free DMEM for 2 h prior to the start of experiments. The cells were subsequently washed with a bicarbonate-buffered physiological salt solution, Gey & Gey buffer (Gey & Gey 1936) containing 2 mM glucose, 2 mM $CaCl_2$ and 0·5 mg/ml BSA, and incubated for 1 h in the salt solution supplemented with agents of interest. The insulin content of the incubation medium was assessed using an in-house radioimmunoassay with an antibody to bovine insulin generated in guinea pigs (Jones *et al.* 1988).

Perifusion experiments were used for measurement of the dynamics of the secretory response. MIN6 pseudoislets were pre-incubated for 2 h in glucose-free DMEM after which equal aliquots of pseudoislets were transferred to perifusion chambers ($\sim$1000 pseudoislets per chamber) essentially as described for primary rat islets (Jones *et al.* 1989, Persaud *et al.* 2002). Pseudoislets exist as free-floating structures on the gelatin substrate, and can be removed and placed in the perifusion chambers by simple pipetting, eliminating any element of dispersal in their preparation for perifusion. In this respect they are handled in the same way as primary islets of Langerhans, and like primary islets they maintain their structure during the perifusion. Human and mouse islets were treated in a similar fashion, and approximately 100 primary islets were used per perifusion chamber because of the higher insulin content of primary tissue compared with MIN6 cells (Hauge-Evans *et al.* 1999). Tissues were perifused for 1 h (0·5 ml/min, 37 °C) with Gey & Gey buffer containing 2 mM glucose to ensure a stable baseline rate of insulin secretion, after which fractions were collected at 2 min intervals and agents of interest were added to the perifusate, as described below.

Detection of ACTH receptor by RT-PCR

Total RNA was isolated from MIN6 cells, mouse brain and mouse and rat adrenal cortex cells using a commercially available kit, RNAzol B. RNA (1–5 μg) was transcribed into cDNA using $oligo(dT)_{18}$ primers, random

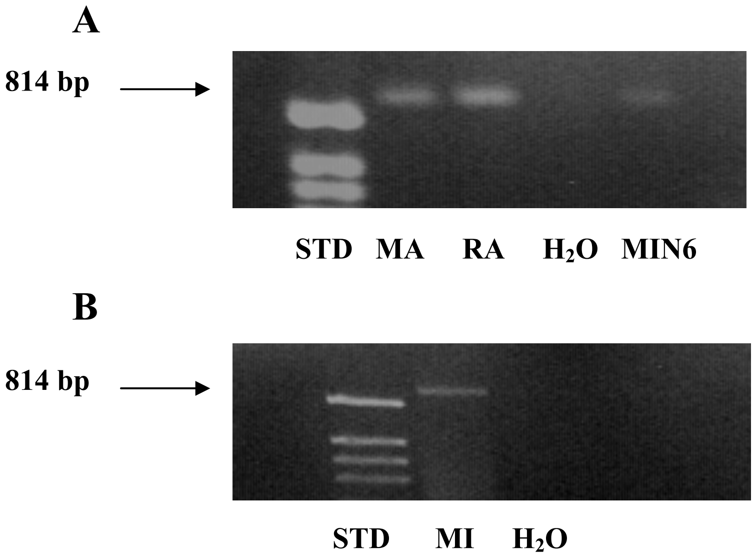

Figure 1 Detection of MC2-R (ACTH receptor) by PCR. Products obtained from RT-PCR amplification are shown after separation by gel electrophoresis. (A) The lanes containing products amplified from cDNA from mouse adrenal (MA), rat adrenal (RA) and MIN6 cells all exhibit a PCR product with a molecular weight corresponding to the expected 814 bp fragment of MC2-R. Rat and mouse adrenal tissues were used as positive controls, water (H_2O) as negative control. (B) The lane containing mouse islets (MI) exhibits a similar sized fragment. Lane 1 (STD) shows molecular weight markers.

10-mer primers and Superscript II reverse transcriptase. PCR was performed using 0·5 μM primers and 1 μl cDNA in a standard reaction mixture containing 100 μM dNTPs, 1 × PCR buffer and 1 unit Taq DNA polymerase added in hot start conditions. The MC2-R cDNA was amplified using oligonucleotide primers designed to amplify a 814 bp product specific for mouse MC2-R. The forward and reverse primer sequences were 5′ AAC TCC GAT TGT CCT GAT GTA G 3′ and 5′ CTT TTG AAT GCA TCT CTG AGC TC 3′ respectively (Boston & Cone 1996). PCR was performed using a final concentration of 0·5 μM of both forward and reverse primers in a standard reaction mixture. Forty cycles of amplification were performed in the presence of 0·5 mM $MgCl_2$ under the following conditions: melting 95 °C for 1 min, annealing 57 °C for 1 min, extension 72 °C for 1 min. PCR products were separated on a 1·5% agarose gel and visualized by ethidium bromide staining. After separation, the PCR products were eluted from the agarose, purified using a QIAquick Gel Extraction Kit and sequenced to confirm their identity (Molecular Biology Unit, King's College London, UK).

cAMP assay

MIN6 monolayers were incubated for 1 h with Gey & Gey containing 2 mM glucose at 37 °C, 5% CO_2, followed by non-tryptic dispersal into cell suspensions to avoid damage to cell surface receptors. Cells were counted, and 50 000 cells were resuspended in 400 μl pre-warmed (37 °C) Gey & Gey buffer containing 100 μM isobutylmethylxanthine (IBMX) in the presence or absence of $ACTH_{1–24}$ (0·1, 1, 10 nM) or 10 μM forskolin (FSK) and incubated for 20 min at 37 °C. After centrifugation to pellet the cells, 100 μl of the incubation medium was added to 400 μl borate buffer for assay of insulin content. The remainder of the supernatant was discarded and 250 μl ice-cold 50 mM Na acetate (pH 6·2) was added to the cell pellet. Samples were then boiled, sonicated and stored at −20 °C until assayed for cAMP content using the method of Harper & Brooker (1975) with acetylation.

Results

Detection of MC2-R expression in MIN6 cells and mouse islets

MIN6 cells expressed RNA species that could be amplified using specific PCR primers for the MC2-R as shown in Fig. 1A. The product amplified from MIN6 cDNA was similar to the product amplified from total RNA extracted from mouse adrenal (MA) and rat adrenal (RA) tissues, which were used as positive controls and the molecular weight of the products corresponded to the expected DNA fragment of 814 bp. A similar product was amplified from mouse islet cDNA (MI), as shown in Fig. 1B. DNA sequencing confirmed that the PCR fragments derived from the mouse islets and MIN6 cells contained the predicted regions of the MC2-R (data not shown).

Effect of $ACTH_{1–24}$ on insulin secretion from MIN6 cells and pseudoislets

$ACTH_{1–24}$ stimulated insulin secretion both from MIN6 pseudoislets in perifusions and from monolayers of MIN6 cells in static incubations. Figures 2A and B show the effect of 1 and 10 nM $ACTH_{1–24}$, respectively, on insulin release from MIN6 pseudoislets that were perifused with buffer containing 2 mM glucose for 10 min before addition of $ACTH_{1–24}$. It can be seen that 1 nM $ACTH_{1–24}$ evoked a significant increase in insulin secretion (186 ± 13% basal, $P<0·01$), a further enhancement of the secretory response was observed when MIN6 pseudoislets were perifused with 10 nM $ACTH_{1–24}$ with a peak rise to 235 ± 11% basal secretion ($P<0·01$, vs 1 nM $ACTH_{1–24}$). The responses were characterized by an initial elevation that reached peak values within 5–6 min of the onset of stimulation and then declined to basal levels after 10–12 min, despite the continued presence of $ACTH_{1–24}$. Increasing the glucose content of the medium to 8 mM caused a rapid and marked increase in insulin secretion from perifused MIN6 pseudoislets (Fig. 2C) with a typical, transient first phase peak (249 ± 15%) that is followed by a sustained second phase of secretion at a significantly lower level (165 ± 15%, $P<0·01$). When $ACTH_{1–24}$ (1 nM or 10 nM; Figs 2D and E) was added to the perifusion medium in the second phase of secretion, there was an elevation of insulin secretion to levels that were not significantly different from those achieved in the first phase.

The effects of $ACTH_{1–24}$ and $ACTH_{1–39}$ on insulin secretion by monolayers of MIN6 cells were tested in static incubation experiments over a 20-minute period. Figure 3A indicates that $ACTH_{1–24}$ (1 pM–10 nM) provoked a concentration-dependent increase in insulin secretion from MIN6 monolayer cells that achieved statistical significance at a concentration of 0·1 nM. Similar responses were obtained with $ACTH_{1–39}$ (0·1 nM–10 nM, Fig. 3B).

Effect of ACTH on β cell cAMP

To determine whether ACTH activated adenylate cyclase in MIN6 cells, intracellular cAMP levels were determined following treatment with FSK (10 μM) or $ACTH_{1–24}$ (0·1 nM and 1 nM). Cells were incubated in buffer containing 2 mM glucose and supplemented with IBMX (100 μM) for 20 min in the absence or presence of stimulants (Table 1). Addition of FSK (10 μM) to the incubation medium evoked a large increase in cAMP levels in MIN6 ($P<0·01$). Addition of 0·1 nM $ACTH_{1–24}$ had no effect on cAMP levels but 1 nM $ACTH_{1–24}$ caused a small, statistically significant increase ($P<0·05$).

Effect of IBMX on the response to ACTH

The phosphodiesterase inhibitor, IBMX (100 μM), potentiated the response of MIN6 cells to sub-maximal doses of $ACTH_{1–24}$, as shown in Fig. 4. A dose-related stimulation of insulin secretion was obtained in the presence of $ACTH_{1–24}$ with a significant potentiation of the response by IBMX being detected at concentrations between 0·1 and 5 nM. Similar results were obtained when cells were exposed to $ACTH_{1–39}$ in the presence of IBMX (data not shown).

Effect of PKA inhibitors on the response to ACTH

The effects of two structurally dissimilar agents that inhibit PKA, Rp-cAMPS (500 μM) and H-89 (10 μM), were studied. Figures 5A and B show that the concentration-dependent stimulation of insulin secretion by $ACTH_{1–24}$ (0·5–10 nM, open bars) was abolished in the presence of either PKA inhibitor.

Importance of extracellular Ca^{2+} in the response to ACTH

Secretory responses of MIN6 cells to $ACTH_{1–24}$ were influenced by changes in extracellular Ca^{2+} levels. MIN6 monolayers were incubated for 20 min with $ACTH_{1–24}$ (1 and 10 nM), in the absence or presence of 2 mM $CaCl_2$ in the incubation medium (Fig. 6). As shown above, insulin release was significantly increased by $ACTH_{1–24}$ when MIN6 cells were incubated in the presence of extracellular Ca^{2+}. However, incubation with $ACTH_{1–24}$ had no effect on insulin secretion in a Ca^{2+}–free buffer supplemented with 0·1 mM EGTA, and the addition of a blocker of L-type voltage-gated Ca2+ channels (nitrendipine, 10μM) to the Ca^{2+}-containing buffer also blocked the secretory response to 1 and 10 nM $ACTH_{1–24}$.

Effects of $ACTH_{1–24}$ on insulin secretion from primary tissue

$ACTH_{1–24}$ evoked an insulin secretory response from perifused mouse islets (Fig. 7). The islets were perifused with buffer containing 2 mM glucose for 10 min followed by perifusion with buffer supplemented with 8 mM glucose alone for 20 min, then followed by 0·5, 1 and 5 nM $ACTH_{1–24}$ for 10 min each in the presence of 8 mM glucose. Insulin release from perifused mouse islets in response to 8 mM glucose was characterized by an initial rapid peak between 2 and 3 min followed by a decline to a sustained plateau above the basal levels after 10–12 min. The addition of 0·5 nM $ACTH_{1–24}$ induced a small and transient increase in insulin secretion from mouse islets at

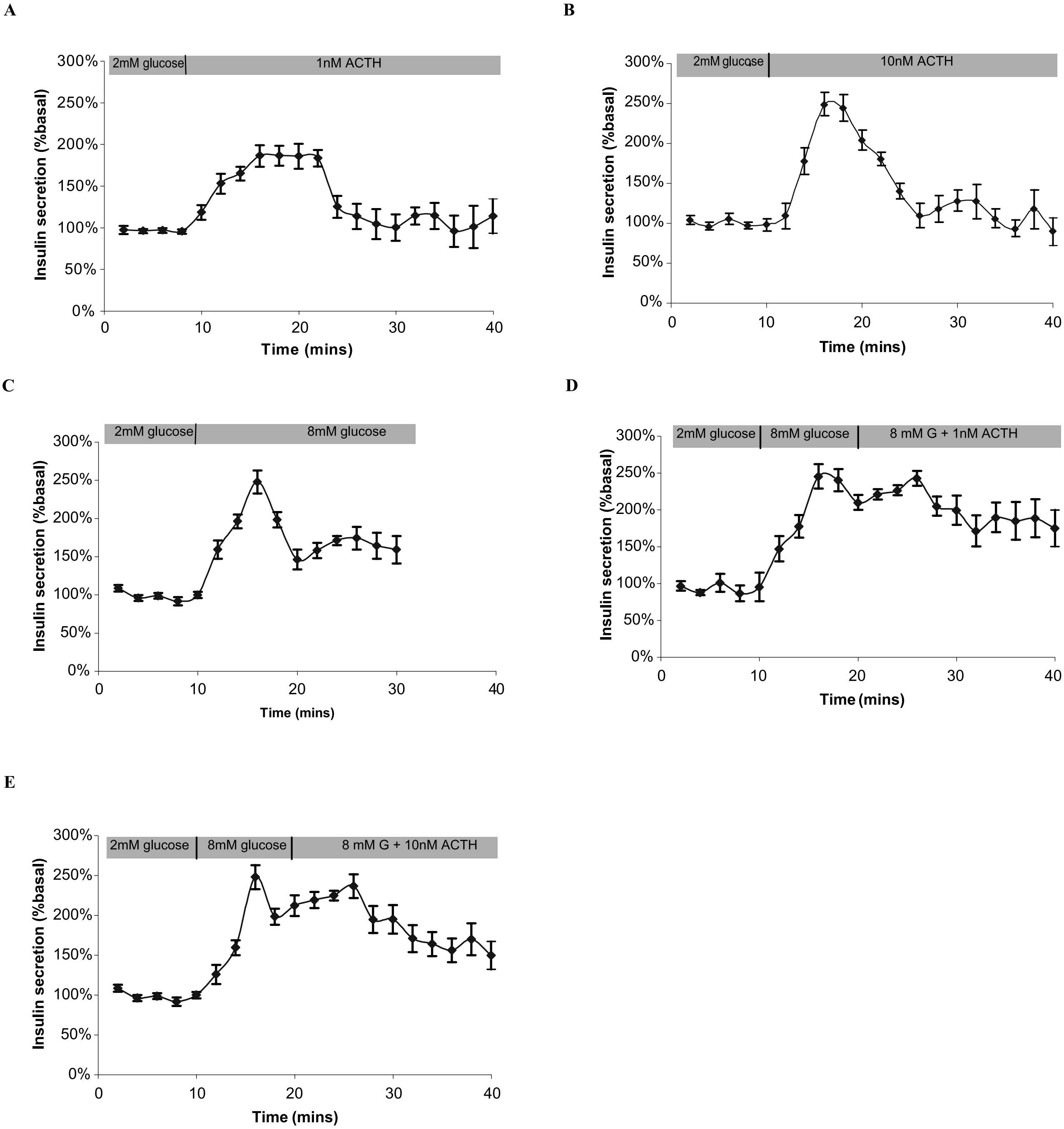

Figure 2 Effect of $ACTH_{1–24}$ on insulin secretion by perifused MIN6 pseudoislets at basal and stimulatory glucose concentrations. MIN6 pseudoislets were perifused with buffer containing 2 mM glucose for 10 min before stimulation: (A) 1 nM and (B) 10 nM $ACTH_{1–24}$ for 30 min; (C) application of buffer containing 8 mM glucose for 20 min and (D, E) application of medium containing 8 mM glucose for 10 min before stimulation with 8 mM glucose (G) and 1 nM (D) and 10 nM (E) $ACTH_{1–24}$ for 20 min. Responses were expressed as percentage of basal insulin secretion in the absence of $ACTH_{1–24}$. Bars showed means ± S.E.M., n=4 channels for each treatment.

8 mM glucose. The presence of higher concentrations of $ACTH_{1–24}$ (1 and 5 nM) in the perifusion medium caused further transient increases in secretion but these were smaller than the initial response.

Figure 8 demonstrates that human primary islets also showed significant secretory responses to $ACTH_{1–24}$ at both sub-stimulatory and stimulatory concentrations of glucose. Human islets were perifused with buffer

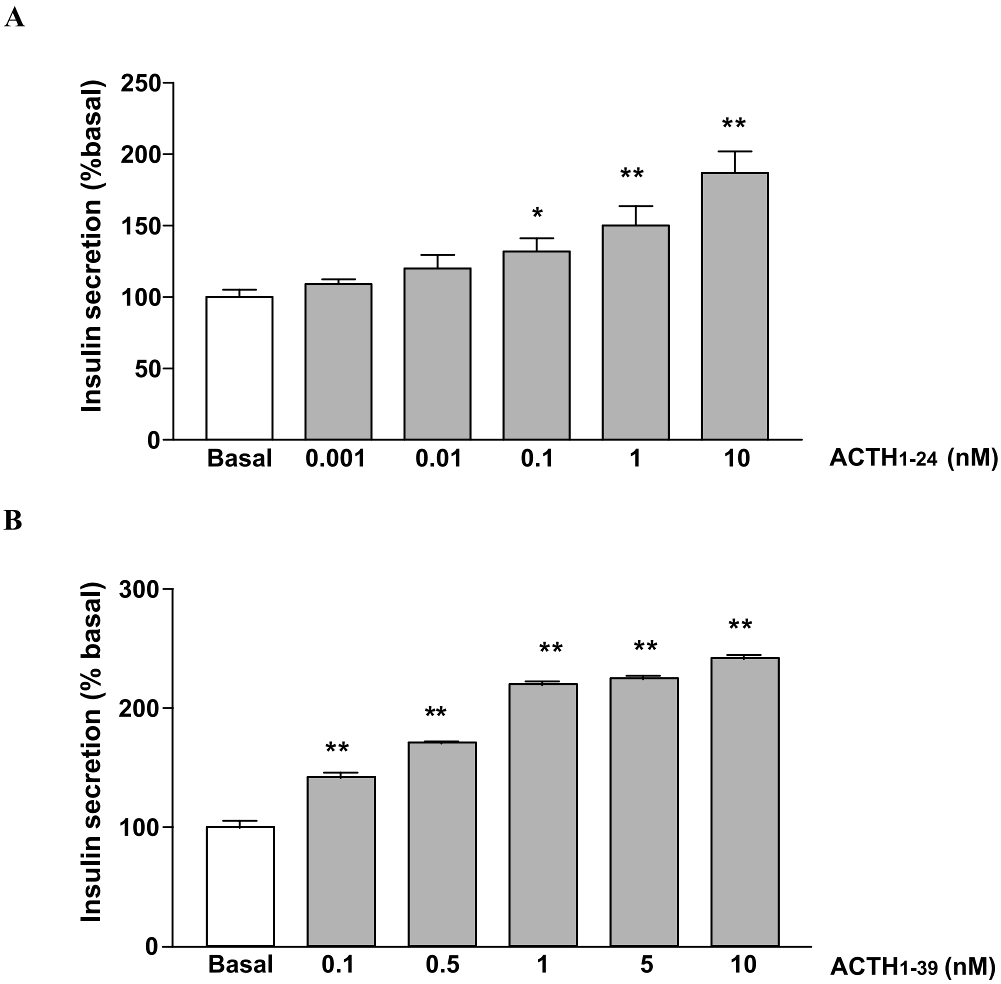

Figure 3 Effects of $ACTH_{1-24}$ and $ACTH_{1-39}$ on insulin secretion by MIN6 cells. MIN6 cells were incubated for 20 min in buffer containing 2 mM glucose in the presence or absence of (A) $ACTH_{1-24}$ (0·001–10 nM) or (B) $ACTH_{1-39}$ (0·1–10 nM). The secretory responses were expressed as a percentage of basal secretion (A, 0·589 ng/30 000 cells/hr; B, 0·851 ng/30 000 cells/hr). Bars represent means ± S.E.M., n=8 observations, **P<0·01 vs basal secretion, *P<0·05 vs basal secretion.

containing 2 mM glucose for 10 min before stimulation with 1 nM $ACTH_{1-24}$ for 20 min followed by 5 nM $ACTH_{1-24}$ for a similar period. At basal glucose concentration (2 mM), the ACTH-mediated secretory response was direct and rapid in onset (2–3 min). The response was characterized by an initial increase in insulin secretion to 319 ± 12·9% of basal secretion followed by a decline to basal levels. A subsequent exposure to 5 nM $ACTH_{1-24}$ did not affect insulin release as markedly, although a slight, transient increase was observed. The response to 1 nM $ACTH_{1-24}$ was also measured at 8 mM glucose, as shown in Fig. 8B. As expected, the stimulatory glucose concentration induced a marked increase in insulin secretion from human islets and 1 nM $ACTH_{1-24}$ caused a further transient increase in insulin secretion when added in the presence of the stimulatory glucose concentration.

Discussion

The results obtained in the present study confirm and extend previous reports of a direct insulinotropic action of pro-opiomelanocortin (POMC)-related peptides by demonstrating that ACTH increases insulin release from both mouse and human primary islet tissue and also from MIN6 cells, a pure β-cell line. Our experiments suggest that the effect of ACTH on insulin secretion from β-cells is mediated through the MC2-R, that it is dependent on activation of PKA, and that it requires Ca^{2+} entry into the β-cells through voltage dependent Ca^{2+} channels.

The results obtained show that pancreatic β-cells express the MC2-R. RT-PCR confirmed the expression of the MC2-R mRNA in mouse islets and the detection of the same mRNA in MIN6 cells suggest that the

Table 1 Effects of FSK and ACTH on intracellular cAMP content of MIN6 cells. MIN6 cells were incubated for 20 min in buffer containing 100 μM IBMX (control) in the presence of 0·1 or 1 nM $ACTH_{1-24}$ or 10 μM FSK. After centrifugation, the cAMP content of the cell lysate was measured by RIA

Treatment	**cAMP** (fmol/20 000 cells/30 min
100 μM IBMX	74·7 ± 2·8
100 μM IBMX+10 μM FSK	233·3 ± 8·1**
100 μM IBMX+0·1 nM $ACTH_{1-24}$	81·7 ± 3·8
100 μM IBMX+1 nM $ACTH_{1-24}$	102·6 ± 7·7*

***P*<0·01, **P*<0·05.

expression occurs in the β-cells of islets of Langerhans. Sequencing of the PCR products confirmed the identity of the predicted regions of the MC2-R. This is the first demonstration of the MC2-R in β-cells, its distribution was initially found to be restricted to the adrenal cortex (Mountjoy *et al.* 1992). Since then ACTH receptors have also been found in low abundance in skin, adipose tissue and, most recently, in fetal testis (Boston & Cone 1996, Slominski *et al.* 1996, O'Shaugnessy *et al.* 2003). Pharmacological studies have indicated that the MC2-R binds ACTH with much higher affinity than other melanocortin-derived peptides (Schioth *et al.* 1996), suggesting that this receptor is responsible for mediating the effects of ACTH in β-cells.

We have shown that ACTH consistently increased insulin release from MIN6 cells in both perifusions and static incubations at a sub-stimulatory glucose concentration. To ensure that this effect represented a true initiation of a secretory response, rather than the amplification of a glucose-induced secretory response (see Ashcroft & Ashcroft 1992), in these experiments the basal glucose concentration was maintained at 2 mM, well below the threshold at which glucose will initiate a secretory response (4–5 mM; Ashcroft & Ashcroft 1992). The naturally occurring hormone, $ACTH_{1-39}$, and a synthetic peptide with full corticotropic activity, $ACTH_{1-24}$ (Baumann *et al.* 1986), were similarly effective as insulin secretagogues in static incubations over doses ranging from 100 pM to 10 nM. In perifusion experiments using MIN6 cells configured as pseudoislets, the response to 1 and 10 nM ACTH at 2 mM glucose was characterized by a rapid increase in insulin secretion that returned to almost basal levels within 15 min in the continued presence of the peptide. The transience of the response to ACTH is not entirely unexpected since the relatively rapid desensitization of β-cell responses to other receptor-operated stimuli has been reported previously (e.g. Squires *et al.* 1994, Kesper *et al.* 1999). This rapid desensitization could also explain the much reduced secretory responses to a second exposure to ACTH that was observed in the present study. A similar progressive decline in insulin secretory responses to repetitive cholinergic activation has been attributed to changes in protein kinase C activation (Verspohl & Wienecke 1998). The responses to ACTH, although still detectable, were partially masked by the marked secretory response to stimulatory concentrations of glucose (8 mM) in the perfusions. Previously, it was generally maintained that receptor-operated insulinotropic stimuli do not initiate insulin secretion, but instead act to enhance the magnitude of secretory responses initiated by nutrients such as glucose (see Ashcroft & Ashcroft 1992, Jones & Persaud 1998). However, our data clearly demonstrate that ACTH can initiate insulin secretion from MIN6 cells and human primary islets at sub-stimulatory

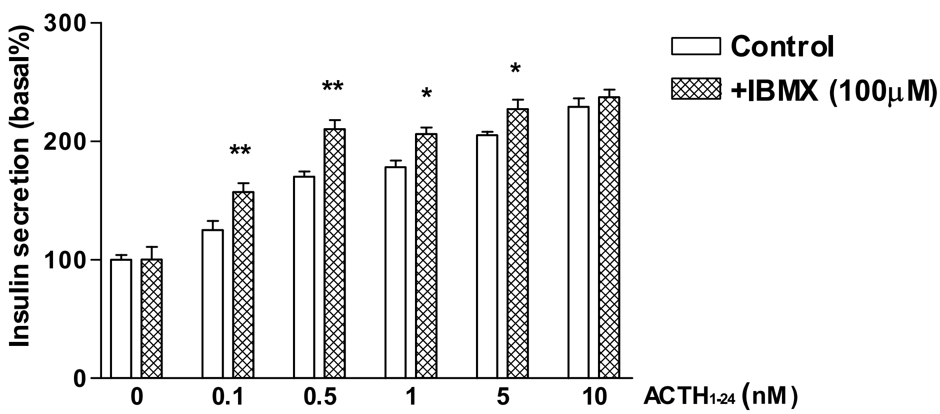

Figure 4 The effect of IBMX on the response of MIN6 cells to $ACTH_{1-24}$. The cells were incubated for 20 min in buffer containing 2 mM glucose in the absence or presence of $ACTH_{1-24}$ (0·1–50 nM) alone or $ACTH_{1-24}$+100 μM IBMX. Data are expressed as percentage of basal secretion: 0·657 ng/30 000 cells/hr. Bars represent means ± S.E.M. of eight observations. IBMX caused a significant potentiation of $ACTH_{1-24}$-induced insulin secretion at all concentrations of $ACTH_{1-24}$. **P*< 0·05 vs no IBMX, ***P*< 0·01 vs no IBMX.

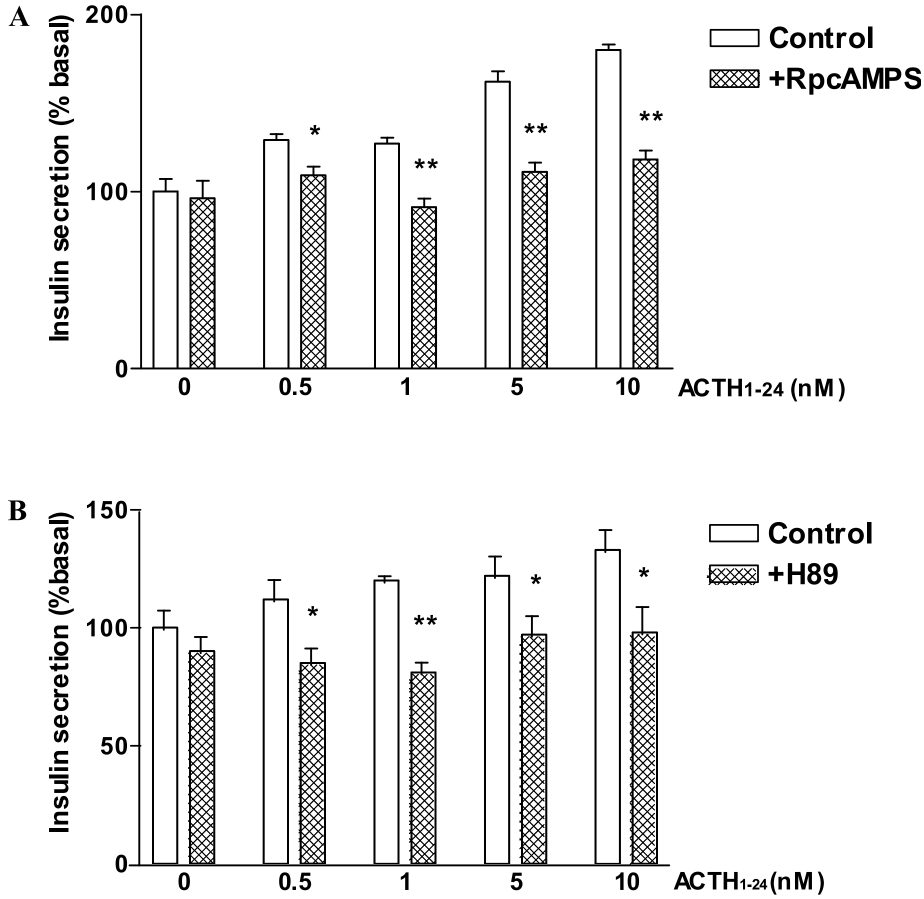

Figure 5 Effect of Rp-cAMPS and H-89 on the response of MIN6 cells to $ACTH_{1–24}$. The cells were incubated with $ACTH_{1–24}$ for 20 min (A) in the presence or absence of the competitive inhibitor Rp-cAMPS (500 µM) or (B) after pretreatment with H-89 (10 µM). Responses were expressed as percentage of basal secretion (A, 0·983 ng/30 000 cells/hr, B, 0·976 ng/30 000 cells/hr). Bars represent means ± S.E.M. of eight observations. Both Rp-cAMPS and H-89 inhibited the secretory responses to 0·5, 1, 5 and 10 nM $ACTH_{1–24}$. $^{**}P<0·01$, $^{*}P<0·05$ vs appropriate $ACTH_{1–24}$ concentrations.

concentrations of glucose. This capacity of ACTH to initiate insulin secretion was also observed in studies with rat islets of Langerhans where exogenous $ACTH_{1–39}$ initiated insulin secretion at a sub-stimulatory glucose concentration (Gronda *et al.* 1992, Borelli *et al.* 1994, 1996, Gagliardino *et al.* 1997). However, in this case ACTH was also found to potentiate glucose-induced (16·7 mM) insulin secretion to a marked extent. Insulin secretion from primary β-cells in islets is modulated by a complex system of endocrine and paracrine signals. Thus, responses in whole pancreas and islet preparations may involve interactions between different cell types that cannot occur in homogeneous β-cell preparations (Pipeleers 1987). Our use of MIN6 cells configured as monolayers and pseudoislets has demonstrated a direct stimulatory action of ACTH on β-cells and provided a model system in which to investigate the mode of action of ACTH in the absence of paracrine influences.

It has long been known that the ACTH action in the adrenal cortex is coupled to activation of the cAMP-dependent PKA signaling pathway (Garren *et al.* 1971, Vinson *et al.* 1992). Early studies with pancreatic preparations implicated cAMP in the effects of ACTH on insulin secretion (Malaisse *et al.* 1967, Sussman & Vaughan 1967, Kuo *et al.* 1973) and the data obtained from the present experiments with MIN6 cells also support this model. First, measurement of the cAMP content of MIN6 cells revealed increased cAMP following stimulation by $ACTH_{1–24}$. Secondly, the phosphodiesterase inhibitor, IBMX, significantly potentiated ACTH-induced insulin release at sub-maximal ACTH concentrations, consistent with the view that ACTH and IBMX are both working by increasing levels of cAMP. Thirdly, the role of PKA in ACTH-induced insulin secretion was confirmed by the use of two structurally and functionally dissimilar PKA inhibitors. Rp-cAMPS is an

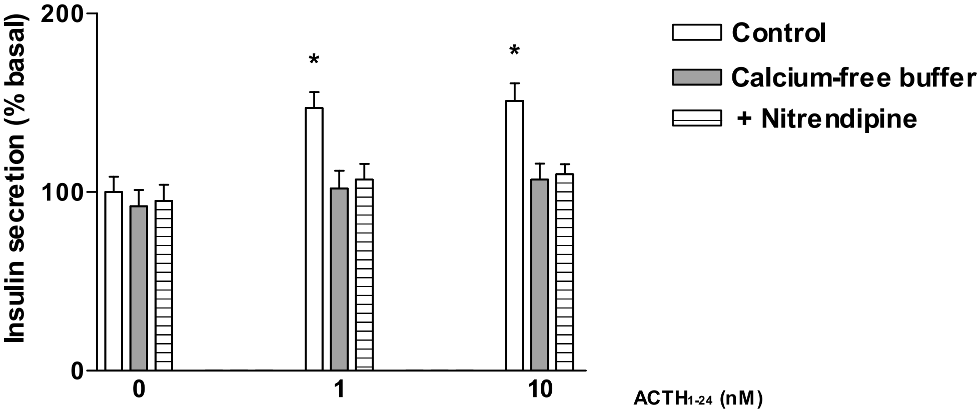

Figure 6 Effect of extracellular Ca^{2+} levels on the response of MIN6 monolayer cells to $ACTH_{1-24}$. The cells were incubated for 20 mins with 1 or 10 nM $ACTH_{1-24}$ either in normal (control) or Ca^{2+}-free buffer, or in the presence of the Ca^{2+} channel blocker, nitrendipine (10 µM). The responses were expressed as percentage of basal secretion (0·872 ng/30 000 cells/hr). Bars represent means ± S.E.M. of nine observations in two experiments. There was a significant increase in insulin secretion in response to $ACTH_{1-24}$ in the presence of extracellular Ca^{2+} but not in its absence from the incubation medium or in the presence of nitrendipine. $^{*}P<0{\cdot}01$ vs no ACTH.

inhibitor which competes with cAMP in binding to the regulatory subunit of PKA (Botelho *et al.* 1988, Persaud *et al.* 1990), while H-89 binds to the ATP-binding site of the PKA catalytic subunit (Chijiwa *et al.* 1990). Both compounds inhibited the insulin secretory response of MIN6 cells to a range of concentrations of ACTH, implicating PKA activation as a major player in transducing the effects of ACTH on insulin secretion.

Agents that increase intracellular cAMP in pancreatic β-cells are generally thought to enhance insulin release at stimulatory, but not at sub-stimulatory, glucose levels (reviewed by Hughes & Ashcroft 1992). However, the present study suggests that ACTH can initiate an insulin secretory response at a sub-stimulatory concentration of glucose, perhaps by activating other intracellular pathways in parallel with the PKA pathway. It has been suggested that ACTH may modulate glucose-induced insulin secretion through pathways coupled to increased cytosolic Ca^{2+} (Gronda *et al.* 1992, Gagliardino *et al.* 1997) and recent studies in the βTC3 and MIN6 cell lines have demonstrated PKA-dependent modulation of Ca^{2+} entry through L-type Ca^{2+} channels (Gao *et al.* 2002, Gomez *et al.* 2002). Our studies support a role for Ca^{2+} entry in ACTH-induced insulin secretion since secretory responses were blocked by the absence of extracellular Ca^{2+} and by the presence of nitrendipine, an L-type voltage-dependent Ca^{2+} channel blocker. These results imply that Ca^{2+} movement from the extracellular space into β-cells is required for ACTH-induced insulin secretion, although the secretory response is primarily PKA-dependent. It seems plausible that ACTH receptor activation will influence the function of β-cells and adrenal cells using similar intracellular transduction systems. Our observations in β-cells are consistent with reports of ACTH action in the adrenal cortex suggesting that stimulation of steroid synthesis by ACTH, although primarily PKA-mediated, also appears to involve a requirement for uptake of extracellular Ca^{2+} (Kojima *et al.* 1985*a*,*b*, Schiebinger *et al.* 1986, Gallo-Payet & Payet 1989). Thus, removal of extracellular Ca^{2+} blocked the steroidogenic effects of ACTH on bovine adrenocortical cells, while the addition of Ca^{2+} restored normal cortisol synthesis (Davies *et al.* 1985). In the present study we were unable to show any ACTH-induced changes in intracellular Ca^{2+} in MIN6 cells by

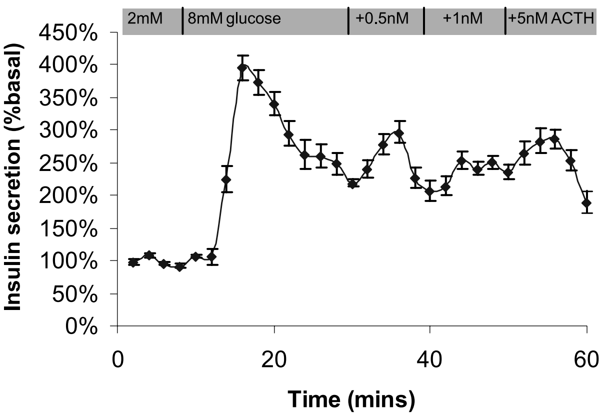

Figure 7 Effect of $ACTH_{1-24}$ on insulin secretion by perifused mouse islets. After perifusion for 1 h with Gey & Gey buffer containing 2 mM glucose to equilibrate insulin secretion, mouse islets were then perifused for 10 min with 2 mM glucose medium before application of 8 mM glucose medium for 20 min. This was followed by perifusion with increasing concentrations of $ACTH_{1-24}$ (0·5, 1 and 5 nM) for 10 min each in the continued presence of 8 mM glucose. Responses were expressed as percentage of basal insulin secretion in the absence of $ACTH_{1-24}$. Bars showed means ± S.E.M., $n=4$ channels for each treatment.

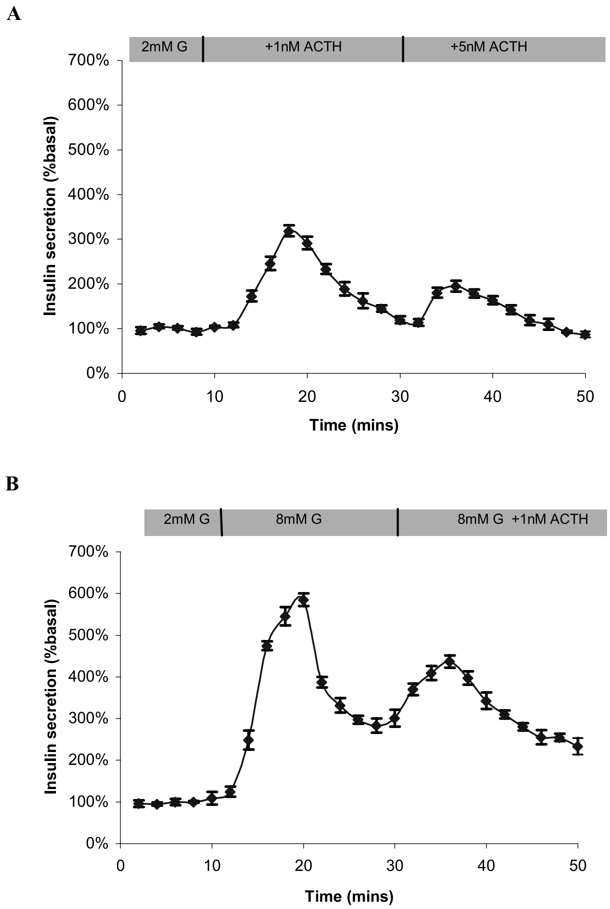

Figure 8 Effects of $ACTH_{1–24}$ on insulin secretion by human primary islets. Human islets were perifused for 1 h with Gey & Gey buffer containing 2 mM glucose (G). This was followed by (A) perifusion with medium containing 2 mM G supplemented with 1 and then 5 nM $ACTH_{1–24}$ for 20 min each, or (B) perifusion with 8 mM G medium for 20 min followed by perifusion with 8 mM G and 1 nM $ACTH_{1–24}$ for 20 min. Secretory responses were expressed as percentage of basal secretion. Bars showed means ± S.E.M., n=4 channels for each treatment.

Fura-2 microfluorimetry (data not shown), but this does not rule out a role for ACTH-induced Ca^{2+}entry into the cells. Thus, in adrenocortical cells, ACTH caused changes in permeability to K^+ that led to Ca^{2+} influx through voltage-dependent Ca^{2+} channels. However, this influx of extracellular Ca^{2+} did not produce sustained increases in intracellular Ca^{2+}, suggesting that ACTH caused an increased flux of Ca^{2+} through the cell (Kenyon *et al.* 1985).

Our experiments using mouse and human islets demonstrate that the ability to respond to ACTH is a property of primary β-cells, suggesting that ACTH receptor activation may play a physiological role in regulating β-cell function. The lowest effective concentration of ACTH (100 pM) in our experiments was somewhat higher than the normal circulating concentration of this peptide (1–10 pM; Estivariz *et al.* 1988). However, there are circumstances under which pancreatic β-cells may be exposed to considerably higher concentrations of ACTH and/or related peptides, for example through local release. It is known that peptides within the pancreas can influence insulin secretion, for example VIP (vasoactive intestinal peptide) and GRP (gastrin releasing peptide) have been localized in nerve terminals within islets and their prime function appears to be to stimulate insulin secretion upon parasympathetic activation (Ahren 2000). It has also been suggested that ACTH or ACTH-like peptides may be similarly involved in the fine control of insulin release; immunoreactive ACTH has been detected within the pancreas and shown to be released from rat islets after glucose stimulation (Larsson 1978, Sanchez-Franco *et al.* 1981, Borelli *et al.* 1994, 1996, Putti *et al.* 1999). In addition, there are diseases, such as ACTH-dependent Cushing's syndrome or congenital adrenal hyperplasia, where concentrations of ACTH in the plasma may be sufficient to affect insulin secretion (Newell-Price *et al.* 1998, Merke *et al.* 2002). However, in both conditions glucocorticoid levels, whether endogenous or exogenous, are also likely to be raised (Charmandari *et al.* 2002). It is difficult, therefore, to assess the contribution ACTH might make to disease pathology against this background, given the known effects of glucocorticoids on insulin secretion and action (Vinson *et al.* 1992).

In conclusion, our studies suggest that ACTH and related peptides can influence β-cell function through activation of the MC2-R, leading to the activation of PKA and increased Ca^{2+} entry through voltage dependent Ca^{2+} channels. The physiological relevance of these effects is most likely to be in the fine control of β-cell function, in a system where ACTH acts along with other biologically active peptides that are released from peptidergic neurons terminating within the islets of Langerhans.

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